v) one or more spacer regions linking any two or more of said i) to iv) components together with one or more formulatory agents.

51. (amended) [A] The pharmaceutical composition according to Claim 50 wherein the formulatory agent is a suspending, preservative, stabilizing [and/or] or dispersing agent.

After the claims please insert the following:

--ABSTRACT

The present invention relates to DNA delivery systems comprising DNA in association with a carrier, said DNA coding for a recombinant chimeric receptor capable of one type of extracellular interaction. In one embodiment, the DNA codes in reading frame for: i) a signal peptide component; ii) an antibody or antigen binding fragment thereof; iii) a transmembrane component; iv) two or more different cytoplasmic signalling components which are not naturally linked, and wherein at least one of said cytoplasmic components is derived from a membrane spanning polypeptide; and optionally v) one or more spacer regions linking any two or more of said i) to iv) components. The invention also relates to effector cells transfected with a DNA delivery system as described herein. The invention further relates to pharmaceutical compositions comprising a DNA delivery system.--

REMARKS

In the Office Action dated March 21, 2000, claims 1-52 are pending in the above referenced application, claims 6-10 and 17-52 stand objected to and claims 1-5 and 11-16 stand rejected. Claims 11, 14, 20-31, 33-42, 46,47, 50 and 51 have been amended to further clarify the subject matter of the invention. Claim 11 has been amended by incorporating the subject matter of claims 13 and 19. Claim 11 is now directed to a DNA delivery system comprising DNA encoding a recombinant chimeric receptor. The DNA encoding the recombinant chimeric receptor comprises an antibody or antigen binding fragment thereof and two or more cytoplasmic signalling components which are not naturally linked. The remaining claims have been

amended in accordance with the amendment to claim 11. Support for the amendments is found throughout the specification and no new matter has been added. Claims 1 to 10, 12, 13, 15 to 19, 32, 43 to 45, 48, 49 and 52, have been cancelled without prejudice. Applicants reserve the right to pursue the subject matter of these claims at a later date, e.g., in a divisional application.

Applicants respectfully request reconsideration of the application in light of the above amendments and the following discussion.

A petition for an extension of time of three (3) months for responding to the outstanding Office Action and the appropriate fee is enclosed herewith.

On Page 2 of the Office Action, the Examiner is requiring a new Declaration that contains a claim for priority to application GB 9526131.9, in order to receive the benefit of the filing date of the GB9526131.9 application. A new Declaration is being prepared and will be submitted to the Patent Office upon execution by the inventors.

The Examiner has pointed out that there are no section headings in the specification. Applicants respectfully submit that these headings are not required and are merely suggested by the MPEP and 37 CFR 1.77. Applicants have amended the specification to include Figures 2a, 2b and 2c and an Abstract.

Claims 6-10 and 17-52 stand objected to under 37 CFR 1.75(c) for improper format and accordingly were not examined on their merits in this Action. Applicants respectfully submit that the above amendments to the claims obviate this rejection. Applicants respectfully submit that the Examiner withdraw the objection and Applicants further submit that the claims are now in condition for allowance.

Claims 1-3 and 11-16 stand rejected under 35 U.S.C. §112, second paragraph, for indefiniteness. Applicants disagree with the Examiner's position. However, in order to expedite examination of this application, the claims have been amended to

further clarify the subject matter of the claims. The Applicants respectfully disagree with the Examiner's position on the following issues.

The Examiner objects to the phrase "one type of extracellular interaction" because the type of interaction is not specified. This phrase however, merely introduces a functional limitation upon the chimeric receptor, namely that it is capable of an extracellular interaction. The precise type of extracellular interaction is not an essential feature of the invention. This is clear from page 2, line 9, page 3, lines 27-31, and page 5, lines 22-30 of the present application. Applicants respectfully submit that one of ordinary skill in the art would recognize what is meant by this term and that it is not necessary to specify the nature of the extracellular interaction.

The Examiner has also objected to the phrase "not naturally linked". The Examiner considers that this term can be interpreted in two different ways. Applicants respectfully disagree with the Examiner and submit that the amended claims clearly indicate that all the elements of the claimed receptor are connected to each other on a single polypeptide chain, unless otherwise stated in the claims. Thus the limitation that two or more cytoplasmic signalling components are "not naturally linked" clearly describes the incorporation of two or more cytoplasmic signalling component into a single polypeptide chain, where they would not normally be found in nature.

Furthermore, it is respectfully submitted that claim 11 is now directed toward DNA that codes, in reading frame, for various components of a chimeric receptor. The term "codes in reading frame" clearly indicates that transcription will result in a single message which in turn is translated to produce a single polypeptide. It is therefore submitted that it would be clear to one of ordinary skill in the art on reading the claim that the cytoplasmic signalling components would be found on a single polypeptide chain, but not linked as they might be found in nature.

The Examiner has objected to the term "derived from". The Examiner has taken the position that this term is unclear when it is used to describe the origin of "at least one of said cytoplasmic signalling components". Applicants respectfully submit that the verb "derive" is defined in dictionaries to mean "to draw" or "to be drawn from" in source or origin. Accordingly, if this standard definition is applied to the claims, it is clear that the source or origin of one of the cytoplasmic signalling components is a membrane spanning polypeptide. In other words, one of the cytoplasmic signalling components of the chimeric receptor is from a membrane spanning polypeptide. Examples of polypeptides from which the cytoplasmic signalling components may be derived are described on page 7, lines 26 to 34. It would readily be apparent to one of ordinary skill in the art that these examples describe membrane spanning polypeptides. Accordingly, Applicants respectfully submit that the meaning of the term "derived from" is clear to one of ordinary skill in the art.

The Examiner has objected to the term "capable of" as indefinite. It is respectfully submitted that the term "capable of" simply applies a functional restriction to the chimeric receptor of claim 11 and a binding component of claim 14. The term clearly indicates that the chimeric receptor "can", or is "able to", take part in one type of extracellular interaction, and the binding component "can", or is "able to", recognize a cell surface molecule. The term defines the claimed subject matter as chimeric receptors and binding components that function as specified and thereby exclude non-functional subject matter.

The Examiner also objects to the term "in association with". It is respectfully submitted that it is not necessary to indicate the nature of the association between the DNA and the carrier in the claims. It would be apparent to one of ordinary skill in the art that the nature of the association would be dependent on the carrier used. Different types of carriers are described on page 9, paragraph 2 (i.e. vectors), and on page 10, paragraph 2 (e.g. target-naked DNA, targeted liposomes encapsulating and/or complexed with DNA). The nature of the association of these carriers will clearly differ. It is not essential to the invention which particular carrier is employed

and it is therefore not necessary to state whether the DNA is associated with the carrier through, for example, chemical bonds or charge-charge interactions.

The Examiner has also objected to the term "separate" when referring to DNA, and has suggested that it is not clear whether separate refers to "separate DNA on the same nucleic acid molecule, or different nucleic acid molecules". Such a distinction is not relevant to the presently claimed subject matter. The invention will work equally well if either of these arrangements are used. Thus it would be understood immediately by one of ordinary skill in the art that the term "separate" as used in this context would mean that a separate message would be transcribed in order to produce a second polypeptide chain. This would occur whether or not the two DNAs are incorporated into the same nucleic acid molecule or different nucleic acid molecules.

Applicants respectfully request that the Examiner reconsider and withdraw the rejections in view of the above amendments and discussion.

Claims 1-5 and 11-16 stand rejected under 35 U.S.C. §112, first paragraph, for lack of enablement. Applicants respectfully traverse this rejection.

The Applicants disagree with the Examiner that operably linked promoters are critical to the invention and should therefore be incorporated as a feature of the claims. While it may be necessary for chimeric receptors of the invention to be expressed in order that they function to activate a cell, they do not have to rely (although they may do so) on promoters that are integral to the DNA delivery system. Alternatively, the DNA encoding the chimeric receptor may recombine with host cell DNA and thus the expression may be mediated by a host cell promoter. The use of integrating vectors, which rely on transactivation of a recombinant gene from a host promoter and/or a regulatory element is well known in the art and would be a straightforward alternative to one of ordinary skill in the art. Applicants therefore respectfully submit that the DNA delivery system of the present application does not require an operably linked promoter. The presence of a promoter is not an essential

feature of the presently claimed subject matter and therefore does not need to be included in the claims.

It is the Examiner's position that the disclosure of the present application does not enable one of ordinary skill in the art to make and use the invention commensurate with the scope of the claims. Applicants respectfully disagree with the Examiner's position. However, in order to expedite examination of this application, Applicants have amended the claims and respectfully submit that the amended claims obviate the rejection. The claims as amended recite chimeric receptors comprising antibodies or antigen binding fragments as the binding component.

Furthermore, the specification clearly provides adequate guidance and teaching as to how to make the invention over the full scope of the claims. Suggested sources and/or methods of production are described for each of the amendments referred to in the claims. For example, even though the production of antibodies and antigen binding fragments thereof are well documented in the art, working examples of scFv fragments are provided in the specification (see Example 1, page 15) and further guidance to the production of engineered human antibodies or fragments thereof is also clearly given by reference to International Patent Application WO 91/09967 (page 6, line 11 of the present application). Similarly the examples of suitable cytoplasmic signalling components, which have also been previously characterized and described fully, are described on page 7, lines 26 to 34 of the specification. Using this information, coupled with that provided in the specific examples (where examples of individual hinge, transmembrane and spacer domains are described) the construction of DNA delivery systems across the scope of the claims would be a facile task to one of ordinary skill in the art of molecular biology.

Applicants therefore, respectfully disagree with the Examiner and submit that the present specification provides sufficient information enabling one skilled in the art to arrive at the subject matter of the present claims. The specification also describes how to introduce the claimed constructs into cells, both in general terms on page 9, lines 27 to 33, and on page 10, lines 8-15, and more specifically in Example 2 on page

23, lines 22 to 29. The present application also describes how to assess whether chimeric rcceptors are expressed (see page 25, lines 6 to 10) and whether or not they stimulate cellular activation, even by the addition of anti-idiotype antibodies (see for example, page 25, lines 12 to 18) or, by stimulation with a natural ligand expressed on the surface of a particular cell typc (sce for example, page 29, line 6 to 16).

These experimcnts and procedures are simple, routine and require no additional intcllectual input by one skilled in the art. By providing the neeessary methodology to assess the functionality of a given receptor construct, it can be seen that the present application clearly provides sufficient teaching to enable one skilled in the art to arrive at the functional chimeric receptors.

On page 10 of the Office Action, the Examiner statcs that there is no precedent for the co-localization of two or more cytoplasmic signalling components resulting in cellular activation. This statement is irrelevant with respect to the question of enablement, but in fact, serves to highlight the novelty of the claimed subject matter. Example 4 on pages 31 to 33 of the present application, demonstrates that the co-localization of two cytoplasmic signalling components within the same cell, though on different receptors, results in cellular activation (IL-2 production) when stimulated with antigen positive cells (see figure 19 and page 33, lines 24 to 27). Furthermore, the results from example 2, described on page 27, line 27 onwards, and as shown in figure 13, illustrates the functional efficacy of a chimeric receptor comprising two cytoplasmic signalling components within the same molecule. It is evident from these examples that co-localization of two or more cytoplasmic signalling components is sufficient to promote or mimic cellular activation.

Despite the extensive teaching in the specification, the Examiner appears to have difficulty in envisioning how these examples are predictive for the full scope of the invention claimed. The Examiner appears to have been misguided by the statement that the physiological art is recognized as unpredictable. This statement is not applicable to the current invcntion for the following reasons.

A review of the art (for example, Weiss and Littman, 1994, Cell, 76, 263-274, and references cited therein) reveals that cytoplasmic signalling components of, for example, the TCR/CD3 complex, B-cell receptor complex (e.g. B27, MB-1) and the gamma chain of the Fc receptor are functionally equivalent. It would therefore be readily apparent to one of ordinary skill in the art that the results described with chimeric zeta chain receptors of the present invention are predictive of those where the zeta chain is substituted for such a functionally similar cytoplasmic signalling component. A copy of the Weiss and Littman article is enclosed herewith for the Examiner's reference.

Similarly, example 5 (see page 34) demonstrates that CD5 and CD2 may be considered functionally equivalent to CD28. The results described for chimeric receptors containing a cytoplasmic signalling component from CD28 are therefore considered predictive for those wherein the CD28 component is substituted for one from CD5 or CD2.

The Examiner has not provided any evidence to demonstrate that the claims encompass chimeric receptors that will not function. On the contrary, the available data clearly indicate that there is a reasonable expectation that the chimeric receptor constructs as claimed will be functional.

It is therefore respectfully submitted that the full scope of the claims of the present application is clearly enabled by the disclosure of the specification. Applicants therefore, respectfully request that the Examiner reconsider and withdraw this rejection.

Claims 1-5 and 11 stand rejected under 35 U.S.C. §102(b) as anticipated by Capon et al. (US Pat. No. 5,359,046). Applicants respectfully traverse this rejection.

Claim 11 as amended is not anticipated by Capon et al. (US-A-5,359,046) for the following reasons. The recombinant DNA described by Capon et al. encodes a signal sequence, an extracellular binding domain, a transmembrane domain and a

single cytoplasmic signalling domain. In contrast, the recombinant DNA of claim 11 encodes a signal sequence, an antibody or antigen binding fragment, a transmembrane domain and two or more cytoplasmic signalling domains. There is a clear difference between the two recombinant DNA molecules, namely that the currently claimed DNA must encode two cytoplasmic signalling components on the same polypeptide chain. Cells transformed with the recombinant DNA described by Capon et al. may also contain further cytoplasmic signalling components, but these are not present in the same polypeptide chain encoded by the recombinant DNA.

The amended claims recite subject matter not disclosed in US-A-5,359,046 and are therefore not anticipated by this reference. Applicants therefore, respectfully request that this rejection be withdrawn.

Claims 1-5, 11-13 and 15 stand rejected under 35 U.S.C. §102(b) as anticipated by Feng et al., J. Biol. Chem. 1995. Applicants respectfully traverse this rejection.

According to Feng et al., TGF-β has two classes of receptors, type I and type II. Feng et al. describes the construction of chimeric receptors comprising the extracellular and transmembrane domains from one class of receptor fused to the cytoplasmic domain from the second class of receptor. The extracellular and transmembrane domains used in each chimeric receptor described in Feng et al. are derived from natural TGF-β receptors and are not antibodies or antigen-binding fragments. Thus the disclosure of Feng et al. does not anticipate claim 11, because claim 11 is directed towards a chimeric receptor where the extracellular domain is an antibody or antigen binding fragment.

Furthermore, Feng et al. only describes a chimeric receptor polypeptide comprising a single cytoplasmic signalling component, whereas the chimeric receptors of claim 11 comprise two or more cytoplasmic signalling components on the same polypeptide chain. This additional distinction between the chimeric receptors of the current invention and those in Feng et al. clearly shows that the two types of receptor

are completely different and, therefore, the present claims are not anticipated by this reference. Applicants therefore, respectfully request that this rejection be withdrawn.

Claims 1-5, 11-13 and 15 stand rejected under 35 U.S.C. §102(b) as anticipated by Nelson et al., Nature 1994. Applicants respectfully traverse this rejection.

Claim 1 as amended recites DNA encoding a chimeric receptor where the binding component is provided for by an antibody or an antigen binding fragment. Nelson fails to teach the presently claimed invention.

Nelson et al. describes chimeric receptors where the extracellular region is derived either from c-kit or GM-CSFR. These regions are derived from natural membrane-bound receptors and act as the binding components in the chimeric receptors described in Nelson et al. It is clear therefore, that such chimeric receptors do not anticipate chimeric receptors where the extracellular binding component is an antibody or antigen binding fragment, which is not derived from a natural membrane-bound receptor, as is the case in the chimeric receptors of the present invention.

The present claims are, therefore, not anticipated by Nelson. Applicants therefore, respectfully request that this rejection be reconsidered and withdrawn.

Claims 1-5 and 11-15 stand rejected under 35 U.S.C. §102(e) as anticipated by Roberts et al. (US Pat. No. 5,712,149). Applicants respectfully traverse this rejection.

Roberts fails to teach the presently claimed invention. Roberts et al. suggests a vast number of possible alternative individual and additional components, and combinations thereof, for all of its chimeric receptors, one of which is illustrated as a speculative cartoon in Figure 1D. However, Roberts et al. do not describe the precise combination of elements as recited in claim 11. One difference is that the extracellular binding domain of the currently claimed invention is provided by an antibody or antigen binding fragment. In complete contrast, the extracellular binding domain of the receptor shown in Figure 1D is provided by the ligand binding domain

of CD4, which is an accessory molecule to foreign antigen recognition in T cells and is not an antibody or antigen binding fragment.

None of the other specific disclosures in the Figures or Examples in Roberts employ an antibody or antigen binding fragment as an extracellular binding domain and it is clear therefore, that the present claims cannot be anticipated by any disclosure contained within US-A-5,712,149. Applicants therefore, respectfully request that this rejection be reconsidered and withdrawn.

Claims 1-5 and 11-16 stand rejected under 35 U.S.C. §102(e) as anticipated by Seed et al. (US Pat. No. 5,912,170). Applicants respectfully traverse this rejection.

Applicants disagree that Seed et al. discloses the present invention. Seed describes the construction of chimeric receptors comprising a single cytoplasmic signalling domain, a transmembrane domain and an extracellular portion of an immunoglobulin superfamily protein. In contrast, chimeric receptors of the present invention comprise more than one cytoplasmic signalling component in the same polypeptide chain. Furthermore, these are not linked to each other as they would be in nature. The Examiner states that Seed describes cells comprising receptors having more than one cytoplasmic signalling component. However, each cytoplasmic signalling component in Seed is present on a separate receptor polypeptide chain. There is no disclosure of a chimeric receptor containing two cytoplasmic components (such as two tyrosine kinases or a tyrosine kinase and a portion of CD28) on the same polypeptide chain, as presently claimed.

Accordingly, Seed et al. does not anticipate the invention as presently claimed. Applicants therefore, respectfully request that this rejection be reconsidered and withdrawn.

The Examiner's reference to claims 32 and 62 (paragraph 1, page 16, of the Office Action) is confusing and unclear. Thus, Applicants have not addressed this

statement by the Examiner. However, Applicants reserve the right to address it at a later time, if the meaning of this statement is further clarified.

Claims 1-5 and 11-16 stand rejected under 35 U.S.C. §102(a) as anticipated by Capon et al. (WO 96/24671). Applicants respectfully traverse this rejection. WO 96/24671 (Capon et al) also fails to anticipate the present invention.

WO 96/24671 describes a multi-specific chimeric receptor that comprises at least three domains that do not exist together naturally. These domains are described as a multispecific binding domain, a transmembrane domain and a cytoplasmic domain, with the binding domain comprising "at least two extracellular inducer-responsive clustering domains (ECD's)" (see claim 1). ECD's are defined further on page 5 of the International Application and, to serve the purpose of the invention, the combination of ECD's must be multispecific (MSECD's) i.e. each ECD must have a different specificity (paragraph 1, page 5).

In contrast, present claim 11 describes recombinant chimeric receptors containing two or more cytoplasmic signalling components, a transmembrane domain and all or part of a (i.e. a single) binding component, which is an antibody or antigen binding fragment. This is clearly different from anything described in WO 96/24671 since that application stipulates that there must be at least two binding components or ECD's. Present claim 14 recites the use of more than one antibody chain, but these chains associate to take part in the same type of binding event. In other words, the component parts of the extracellular binding component of the presently claimed invention are monospecific, i.e. have the same specificity (see also page 6 lines 16 to 24 and page 3 lines 26 to 31 of the specification). This is in contrast to the receptor in WO 96/24671, which must be multispecific.

Furthermore, amended claim 14 recites separate DNA sequences when using separate parts of a binding component, whereas the receptor described by WO 96/24671 is a single polypeptide encoded by a single DNA. It is quite clear, therefore, that the present invention is novel, and not anticipated by WO 96/24671.

Applicants therefore, respectfully request that this rejection be reconsidered and withdrawn.

In view of the amendments and discussion above, it is respectfully submitted that the present application is in condition for allowance. An early reconsideration and notice of allowance are earnestly solicited. Should the Examiner wish to discuss the above amendment made herein, the undersigned attorney would appreciate the opportunity to do so. Thus the Examiner is hereby invited to call the undersigned, collect at the number shown below.

Respectfully submitted,

Date: September 21, 2000

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Review

Signal Transduction by Lymphocyte Antigen Receptors

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Introduction

B and T lymphocytes, the antigen-specific cells of the immune system, are generally quiescent and require antigen stimulation to progress from the G_0 stage of the cell cycle. Specific responses to pathogens or foreign substances require that receptors on these cells recognize the antigen as well as initiate a series of signal transduction events. These signals are fundamental both in developmental decisions and in the initiation of immune responses by B and T cells. Here we focus on recent progress in understanding how T cell antigen receptors (TCRs) initiate signal transduction events that lead to cellular responses. Where appropriate and possible, parallels and contrasts with the B cell system will be discussed.

T and B cells have structurally different oligomeric antigen receptors that recognize fundamentally distinct forms of antigens. B cells function to protect the host from extracellular pathogens. Their receptors typically recognize native or denatured forms of proteins or carbohydrates in soluble, particulate, or cell-bound form. In contrast, the T cell system protects the host against intracellular pathogens. TCRs recognize proteolytically processed short (8–15 residues) peptide antigens bound to self major histocompatibility complex (MHC) molecules on the surface of an antigen-presenting cell. Thus, B and T cells recognize distinct forms of antigen using very different receptors. Remarkably, the signal transduction events that result from the interaction of their antigen receptors with antigen are quite similar.

In addition to the antigen receptors, other molecules contribute to cell activation: first, by functioning as coreceptors (e.g., CD4, CD8, CD19/CD21); second, by increasing the avidity of the interaction with antigen or the antigen-presenting cell (e.g., LFA-1); or, third, by inducing separate signal transduction events that influence the cellular response (e.g., CD28, CD40). Coreceptors, such as CD4 or CD8 in T cells or CD19/CD21 in B cells, are of particular interest in the context of antigen receptor signal transduction, since they directly contribute to the formation of the complex between the antigen receptor and the antigen complex, thereby increasing the sensitivity of the interaction. The coreceptors also contribute to the initiation of signals, as we will discuss below.

Protein-tyrosine phosphorylation is important in the initiation of cellular responses by antigen receptors on either B or T cells. Neither the TCR nor the B cell antigen receptor (BCR) has intrinsic PTK activity. Both appear to activate cytoplasmic PTKs, although at least one tyrosine phosphatase, CD45, is also important in regulating antigen receptor–induced signal transduction. Members of two distinct classes of PTKs, of the Src family and of the Syk/ZAP-70 family, have been implicated in TCR and BCR signal transduction. These distinct PTKs interact with the TCR and BCR as well as with each other.

The events downstream of protein-tyrosine phosphorylation following TCR or BCR stimulation include the activation of the phosphatidylinositol pathway, activation of Ras, and activation of several serine/threonine protein kinases and phosphatases. These events have been causally related to a variety of responses, of which the best characterized is the transcriptional induction of the interleukin 2 (IL-2) gene in T cells. Our understanding of the detailed intermolecular connections between the early events and later cellular responses is rapidly evolving.

The interpretation of the signal transduced by the antigen receptor may differ, depending on the developmental stage of the responding cell or on the cellular context of antigen recognition. For instance, signal transduction by the TCR in developing thymocytes can lead either to programmed cell death (apoptosis) or to selection, depending on the antigen specificity of the TCR. In mature T cells, TCR recognition of antigen can lead either to differentiation and proliferation or to a long-lived state of unresponsiveness (anergy), depending on whether appropriate second (costimulatory) signals are provided by molecules such as CD28, which interacts with ligands on antigen-presenting cells. This review will focus on the biochemical events induced by the antigen receptors, whereas other reviews in this issue will address the different responses that lymphocytes can make to these signals, depending on developmental and cellular contexts.

T and B Cell Antigen Receptors

The ability to recognize a vast array of pathogens and foreign substances is the responsibility of antigen-specific receptors expressed on T and B cells. Both the BCR and TCR have separate antigen-binding and signal transduction subunits (Figure 1). The antigen-binding subunits cannot be expressed independently at the plasma membrane, but must form a complex with the invariant chains responsible for receptor signaling function (Weiss, 1991). The TCR Ti subunit is noncovalently associated with the CD3δ, CD3ε, and CD3γ chains and a ζ chain–containing dimer (which may consist of homodimers or heterodimers with η or FcεRIγ chains). It is believed that each Ti subunit is associated with a CD3εδ and a CD3εγ dimer, though the precise stoichiometry of the chains within the receptor is still not known. The cytoplasmic domains of the associated invariant chains are considerably larger (40 to 113 residues) than those of the Ti α and β chains (5 residues) and are responsible for coupling the Ti subunit to the intracellular signaling machinery.

The BCR membrane immunoglobulin (Ig) subunit is sim-

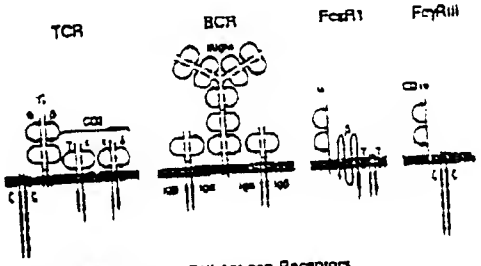

Figure 1. Hematopoietic Cell Antigen Receptors

ARAMs are shown as black rectangles.

ilarly expressed at the plasma membrane in a stable complex with the invariant disulfide-linked Igα (mb-1) and Igβ (B29) subunits (Reth, 1992). The number of heterodimers associated with each membrane Ig subunit within a BCR is not known. Like the CD3 and ζ subunits, the Igα and Igβ chains have more extensive cytoplasmic domains than the antigen-binding membrane Ig chains and couple the BCR to the cytoplasmic signaling apparatus

The signal transduction functions of the invariant nonantigen-binding chains of these receptors were revealed by studies with mutant cell lines (Wegener et al., 1992) or with chimeric receptors, in which the cytoplasmic domains of the ζ, η, CD3 ε, Igα, Igβ, and FcεRIγ chains were linked to the extracellular and transmembrane domains of other proteins (Irving and Weiss, 1991; Kim et al., 1993, Law et al., 1993; Letourneur and Klausner, 1992; Romeo and Seed, 1991). These chimeric receptors could be expressed independently of the TCR, BCR, or FcεRI, and their cross-linking induced early and late signal transduction events characteristic of those observed with intact oligomeric receptors.

The apparent redundancy of function of these invariant chains is explained, as was recently reviewed (Weiss, 1993), by the presence of a common cytoplasmic domain sequence motif that couples these proteins to intracellular PTKs. The minimal functional segment of the motif, first noted by Reth (1989), consists of paired tyrosines and leucines in the consensus sequence (D/E)XXYXXL(X)$_{6-8}$YXXL and will be referred to herein as the antigen recognition activation motif (ARAM), which has also been referred to as the tyrosine-based activation motif (TAM) (Samelson and Klausner, 1992) or the antigen receptor homology 1 (ARH1) motif (Cambier, 1992). The ARAM sequence alone is sufficient to couple chimeric receptors to early and late signaling events (Irving et al., 1993; Letourneur and Klausner, 1992; Romeo et al., 1992).

One of the most striking features of the hematopoietic antigen receptors depicted in Figure 1 is the separation of the signal transduction module containing ARAMs from the ligand-binding module. The common exon–intron organization of the genes encoding the ARAM motif in the invariant chains of the TCR, BCR, and FcεRI suggests a common evolutionary origin (Wegener et al., 1992). Interestingly, two viruses involved in B cell transformation, the Bovine Leukemia Virus (BLV) and Epstein–Barr virus (EBV), also contain functional ARAM sequences in the cytoplasmic domains of the envelope glycoprotein gp30 and the latent membrane protein 2 (LMP2), respectively (Alber et al., 1993). Thus, these two viruses may have usurped the signal transduction module of hematopoietic cell antigen receptors to induce B cell activation.

The presence of multiple ARAM sequences within a single oligomeric receptor raises the possibility that individual ARAMs may interact with distinct signaling molecules. It is unclear, however, whether the different patterns of tyrosine phosphoproteins that have been observed reflect quantitative, rather than qualitative, effects (Letourneur and Klausner, 1992). Multimerization of ARAMs may also provide a means of signal amplification. The immune system must be exquisitely sensitive to low doses of antigen to protect the host from pathogens. It has been estimated that only a few hundred relevant peptide antigen/MHC molecule complexes are present on an antigen-presenting cell (Harding and Unanue, 1990). Thus, relatively few TCRs are engaged by ligand. For this to induce a cellular response, it is likely that the signal transduction events mediated by these few receptors need to be amplified. In support of this, construction of a chimeric receptor with three copies of one of the ζ ARAMs yielded chimeric receptors that were more active than chimeras containing a single copy of the ARAM (Irving et al., 1993; Wegener et al., 1992).

The TCR and BCR Interact with Distinct Families of PTKs

Neither the TCR nor the BCR subunits have intrinsic PTK or PTPase function. Since ARAM sequences are necessary and sufficient for the induction of protein-tyrosine phosphorylation in T cells, it is likely that the ARAMs interact directly with cytoplasmic PTKs. CD3 and ζ chains or chimeras containing their cytoplasmic domains coimmunoprecipitate with PTK activity from T cells or from heterologous cells that have been transfected with appropriate chimeric proteins and cytoplasmic PTKs (Burgess et al., 1991; Chan et al., 1991; Chan et al., 1992; Gauen et al., 1992; Wange et al., 1992). The tyrosines in the ARAMs are required for the induction of PTK activity and are themselves phosphorylated by cellular PTKs (Irving et al., 1993; Letourneur and Klausner, 1992). Similarly, PTK activity has been coprecipitated with the BCR components (Hutchcroft et al., 1991). Moreover, bacterially expressed fusion proteins derived from the BCR Igα and Igβ sequences can associate with cellular PTKs (Clark et al., 1992). Thus, the critical role of the ARAMs may be to function as substrates for cytoplasmic PTKs, thereby allowing the recruitment of additional effector molecules to the stimulated receptor via SH2 domain–phosphotyrosine interactions.

Two classes of cytoplasmic PTKs, members of the Src and Syk/ZAP-70 families, have been implicated in the functions of the TCR and BCR. These classes of PTKs differ in ways that are likely to reflect their distinct functions. Src family members have a unique N-terminal domain with a myristylated glycine at position 2, which is responsible for membrane association. In contrast, Syk and ZAP-70 are not myristylated and hence are probably not constitutively localized at the plasma membrane. The Syk/ZAP-70 family members have two N-terminal SH2 do-

mains and a C-terminal catalytic domain, but lack an SH3 domain and the C-terminal negative regulatory site of tyrosine phosphorylation, which are characteristic of Src family members.

Three Src family members are generally expressed in most T cells: Lck, Fyn, and Yes. At present, the best documented role for a Src family member in signal transduction in T cells is that of Lck. This PTK interacts at high stoichiometry with the cytoplasmic domains of either of the coreceptors, CD4 or CD8 (see below) (Rudd, 1990; Veillette et al., 1991). Although an increase in Lck kinase activity following TCR stimulation has been reported (Danielian et al., 1992), the increase that follows CD4 or CD8 cross-linking has been more consistantly observed (Veillette et al., 1991). Since CD4 or CD8 coengage MHC molecules with the TCR during antigen recognition, Lck would be brought close to ARAMs within the TCR, possibly resulting in their phosphorylation. Consistent with this, antibody cross-linking of CD4 with the TCR potentiates tyrosine phosphorylation of cellular proteins (Ledbetter et al., 1990).

Lck appears to be able to interact with the TCR chains independently of CD4 and CD8. Studies of an Lck kinase-deficient mutant cell line show that the CD3- and ζ-associated PTK activity is dependent upon Lck (Straus and Weiss, 1992, 1993). In NK cells, Lck can interact with the ζ chain in the FcRγIII (Salcedo et al., 1993). Thus, Lck may have an intrinsic ability to interact with ARAMs in ζ or CD3 chains that can function as its substrates. Alternatively, TCR ligation or cross-linking could induce inter- or intramolecular aggregation and/or allosteric changes of the ARAMs, rendering them more accessible substrates for membrane-associated Lck. TCR aggregation, in the absence of coreceptor engagement, could shift an equilibrium between Lck and cellular phosphatases that act on ARAMs to favor phosphorylation of the tyrosines within the ARAMs. Phosphorylation of the ARAMs could then lead to recruitment of other molecules, such as ZAP-70 (see below), involved in signal transduction.

The functional significance of Lck in TCR signal transduction is most clearly indicated by genetic studies in mice and in cell lines. Mice deficient in Lck or expressing a dominant negative Lck transgene have an arrest early in thymocyte development, and the few peripheral T cells that do develop have diminished responses to TCR stimulation (Levin et al., 1993; Molina et al., 1992). In a T cell line deficient in Lck kinase function or a T cell clone deficient in Lck, early and late events associated with TCR signal transduction are abrogated (Karnitz et al., 1992; Straus and Weiss, 1992). Conversely, overexpression of an activated form of Lck (the Y505F mutant in the negative regulatory site) in a CD4-deficient T cell hybridoma potentiates TCR signal transduction in a manner analogous to the effect observed with expression of CD4 in the same cell (Abraham et al., 1991). This effect requires that the Lck mutant has intact kinase function. Collectively, these studies suggest that Lck plays an important function in the initiation of TCR signal transduction.

Genetic and biochemical studies also suggest a role for Fyn in signal transduction in T cells. Fyn PTK activity increases (2- to 4-fold) in response to TCR stimulation (Tsygankov et al., 1992). Using sensitive in vitro kinase assays and mild detergents for solubilization, Fyn PTK activity has been detected in TCR immunoprecipitates, although the association is of low stoichiometry (Samelson et al., 1992). In mice in which the *fyn* gene has been disrupted by homologous recombination, no gross T cell developmental alterations were observed, suggesting that the events associated with TCR-mediated positive and negative selection can occur in the absence of Fyn (Appleby et al., 1992; Stein et al., 1992). Thymocytes in such mice showed a TCR signaling defect that was most pronounced in the mature subset, but was less so in the immature TCR$^+$ thymocytes and peripheral T cells. These observations suggest that Fyn may play an important role in TCR signal transduction in a developmentally restricted T cell compartment. Consistent with these findings, Fyn has been shown to interact functionally with the TCR complex. As was shown with Lck, overexpression of an activated form of Fyn (the negative regulatory tyrosine was mutated to phenylalanine) increased the sensitivity of a T cell hybridoma to TCR stimulation (Davidson et al., 1992). Increased expression of wild-type Fyn in the thymocytes of transgenic mice also caused hyperresponsiveness to TCR stimulation, whereas a kinase-deficient form of Fyn was inhibitory (Cooke et al., 1991). Thus, Fyn appears to be involved in TCR signal transduction in at least some cells.

As in T cells, several Src family members, Lyn, Blk, Fyn, and Lck, have been reported to associate with the BCR (Campbell, 1992). This association appears to involve the ARAMs of the Igα and Igβ chains. Such associations with the TCR or BCR could result from an interaction between a Src family PTK and its substrate (tyrosines in the ARAM) or an interaction between the SH2 domain of the PTK and a small percentage of ARAMs that may be tyrosine phosphorylated in the basal state (Nakayama et al., 1989; van Oers et al., 1993a).

Whereas the nature of the interaction of Src family kinases with TCR and BCR complexes is ill-defined, the association between Syk or ZAP-70 and ARAM-containing receptors is well established. ZAP-70 is a 70 kd PTK expressed exclusively in T cells and NK cells (Chan et al., 1992), whereas Syk, a 72 kd PTK, is expressed preferentially in B cells, myeloid cells, and thymocytes (Taniguchi et al., 1991). ZAP-70 is not associated with the TCR in the basal state, but is rapidly recruited to the ζ and CD3 chains following TCR stimulation (Chan et al., 1991). It only associates with the tyrosine-phosphorylated forms of ζ that are found within the stimulated fraction of receptors. Studies with chimeric receptors show that ZAP-70 requires the ARAMs to associate with ζ (Irving et al., 1993). Thus, the structural features required for ARAM function are the same elements required for ZAP-70 association. It is likely that the ZAP-70 SH2 domains are responsible for recruiting ZAP-70 to tyrosine-phosphorylated ARAMs. Consistent with this, ZAP-70 SH2 domains expressed as bacterial fusion proteins interacted with phosphorylated CD3 and ζ chains from lysates of stimulated T cells (Wange et al., 1993).

These results suggest that the TCR interacts with two

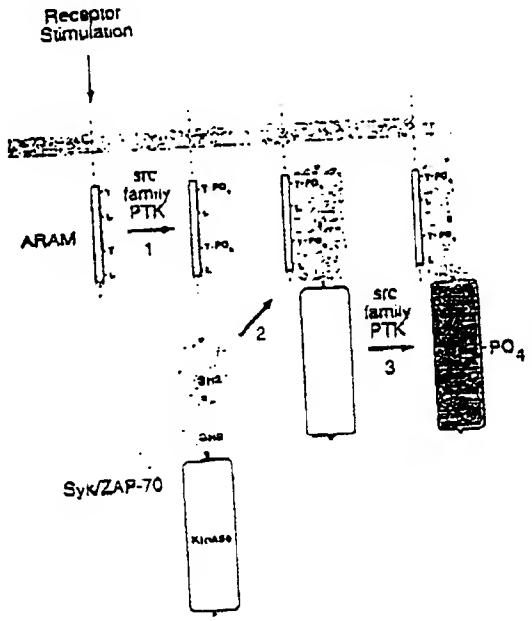

Figure 2. Hypothetical Model of the Recruitment of Syk/ZAP-70 PTKs to ARAMs via a Src PTK Dependent Mechanism

Receptor stimulation with or without coreceptor coengagement results in the phosphorylation of ARAM residues by a Src family PTK to initiate antigen receptor signal transduction.

PTKs sequentially (Figure 2). One of these, probably a Src family member, first phosphorylates the ARAM, resulting in recruitment of ZAP-70, via its SH2 domains, to the membrane receptor complex. This model is supported by studies in a heterologous Cos cell system, in which the association of ZAP-70 with cytoplasmic ζ sequences of a chimeric receptor requires the presence of either Lck or Fyn (Chan et al., 1992). The expression of either of the Src PTKs, but not ZAP-70, alone in this system results in ζ phosphorylation, albeit at low stoichiometry. This is consistent with at least one function of Lck (or Fyn) lying upstream of ZAP-70 in TCR signal transduction.

In B cells, Syk has also been coimmunoprecipitated with components of the BCR complex (Hutchcroft et al., 1991). Chimeric receptors containing Igα and Igβ cytoplasmic sequences can be induced to interact with Syk (Law et al., 1993). Thus, Syk may interact with the BCR complex in a manner analogous to the interaction of ZAP-70 with the TCR.

The mechanism responsible for the induction of PTK activity following Syk/ZAP-70 recruitment to the receptor complexes is unclear. It is likely that additional interactions between the Src and Syk/ZAP-70 families of PTKs mediate the increased catalytic activity observed following receptor stimulation. Activation of Lck alone, for example by cross-linking CD4 or CD8, fails to mimic TCR stimulation (Ledbetter et al., 1990; Veillette et al., 1991). This raises the possibility that the interaction of Lck with the TCR and another kinase, for example ZAP-70, is important. Recent studies with chimeric receptors in which Lck, Fyn, ZAP-70, or Syk were fused to transmembrane proteins support such a model (Kolanus et al., 1993). Aggregation of either Lck or Fyn chimeras failed to induce detectable signal transduction, but aggregation of either Syk or ZAP-70 chimeras induced calcium increases (an indicator of PLC activation), and stimulation of Syk chimeras induced protein-tyrosine phosphorylation. Interestingly, the induction of protein-tyrosine phosphorylation by the ZAP-70 chimera, but not the Syk chimera, required coaggregation with the Fyn chimera (the Lck chimera was not tested). These observations are consistent with the finding that Cos cells transfected with ZAP-70 also require Lck or Fyn for synergistic induction of PTK activity (Chan et al., 1992). Collectively, these studies suggest that Syk and ZAP-70 have roles in later signal transduction events and that the function of ZAP-70, at least, is regulated by Lck or Fyn.

ZAP-70 function may be regulated by Lck or Fyn in several ways. First, as discussed, its recruitment to the stimulated TCR is dependent upon Lck, or perhaps, in some T cells, upon Fyn (Chan et al., 1992). This probably involves ARAM phosphorylation. ZAP-70 is also tyrosine phosphorylated following TCR stimulation (Chan et al., 1991). This could be due to its direct or indirect phosphorylation by Lck or Fyn, or to autophosphorylation. The effect of phosphorylation on the function of ZAP-70 is not yet known. The interaction of the SH2 domains of ZAP-70 with tyrosine phosphorylated ARAMs could, via allosteric changes, induce ZAP-70 kinase activity, or could change substrate availability by its redistribution [illegible] plasma membrane. Finally, there are as yet no data that rule out the possibility that ZAP-70 could also regulate the activity of the Src family PTKs. These complex interactions may also occur between Syk and Src family PTKs.

Coreceptors Contribute to Antigen Receptor Initiation of Signal Transduction Events

Coreceptor Function in T Cells

Most encounters of lymphocytes with antigen are thought to involve low affinity interactions and low occupancy of the antigenic receptors. This handicap may be especially severe in primary immune responses prior to clonal expansion of cells with high affinity receptors. Both B and T lymphocytes handle this problem by employing coreceptor molecules that act synergistically with the antigen receptors to induce signal transduction events at low levels of receptor occupancy. Mechanisms underlying signal amplification have been most thoroughly scrutinized in T lymphocytes, whose integral membrane glycoproteins, CD4 and CD8, serve the essential coreceptor function. Both of these molecules are expressed on immature TCR-bearing thymocytes, but when the thymocyte matures expression of one of the molecules is shut off. Cells that retain CD4 have TCRs specific for MHC class II and have primarily helper functions, whereas cells that retain CD8 expression recognize class I molecules and are almost exclusively cytotoxic.

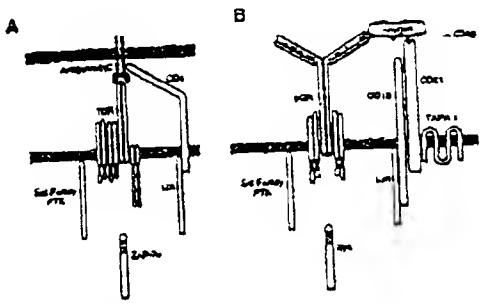

Figure 3. Coreceptor Functions

Coreceptors on T cells (panel A) and B cells (panel B). Signal transduction may be initiated by Src family PTKs that are associated or not associated with coreceptors.

CD4 and CD8 have two key elements that endow them with properties required of a coreceptor (Figure 3). First, their extracellular domains bind to membrane-proximal regions of MHC molecules on antigen-presenting cells: CD4 binds to the β2 segment of MHC class II, whereas CD8 binds to the α3 segment of MHC class I (Cammarota et al., 1992; Konig et al., 1992). These interactions permit simultaneous binding of both TCR and CD4 or CD8 to the same MHC molecule, a requirement for coreceptor function in development and in T cell activation (Wallace et al., 1993). Second, as mentioned above, both CD4 and CD8 bind to the cytoplasmic protein-tyrosine kinase Lck through a cysteine-containing motif shared by their cytoplasmic domains (Veillette et al., 1991). This interaction is essential for effective signal transduction at low antigen concentration in coreceptor-dependent systems (Glaichenhaus et al., 1991; Zamoyska et al., 1989).

Recent studies suggest that Lck has two distinct functions in coreceptor-assisted T cell activation. One of these functions, which may occur independently of coreceptor, involves localization of enzymatically active Lck in close proximity to the TCR complex, where it acts early in the signaling pathway, most likely phosphorylating tyrosines within the ARAMs of the CD3 and ζ chains as discussed above. The availability of Lck for interaction with the TCR complex can determine the responsiveness of the receptor. For example, in some T cells the pool of Lck molecules may be limiting and, hence, may be sequestered from the TCR by interactions with CD4 or CD8 (Haughn et al., 1992; van Oers et al., 1993b). Under such circumstances, stimulation of the TCR in the absence of coreceptor engagement results in diminished responses. Similarly, the diminished TCR responses of single positive thymocytes in Fyn-deficient mice may reflect the lack of available Lck because of sequestration by CD4 or CD8. Fyn, instead of Lck, may then represent the predominant membrane-associated Src kinase that is accessible to the ARAMs when the TCR is stimulated in the absence of coreceptor engagement.

The requirement for Lck association with the TCR may also explain the phenomenon known as CD4-mediated negative signaling, or inhibition of T cell activation when CD4 is cross-linked with antibodies prior to TCR stimulation. Indeed, apoptosis of T cells has been observed under these circumstances, and it has been suggested that this may be a mechanism for HIV-mediated CD4 T cell depletion as a consequence of viral envelope binding to CD4 (Banda et al., 1992). Whether negative signaling involves sequestration is, however, still not clear. Negative signaling may reflect a priming effect of the activation of Lck kinase function prior to TCR signaling events. Alternatively, it could depend on other activities associated with Lck, such as phosphatidylinositol 3′ kinase (PI 3′ kinase), which has been shown to bind to the SH3 domains of Src family members (Liu et al., 1993; Prasad et al., 1993).

The second function of Lck in coreceptor-assisted T cell activation is to coordinate interaction of the TCR and coreceptor with a single MHC molecule. At what normally may be limiting concentrations of CD4, this permits the coreceptor to ignore irrelevant MHC molecules and to be focused only at the site of the TCR–antigen/MHC interaction, thus contributing to the overall avidity of the interaction and facilitating signal transduction. Early studies suggested that CD4 becomes associated with the TCR complex after treatment of T cells with anti-TCR or anti-CD3 antibodies (Saizawa et al., 1987). This association was found to require an intact CD4 cytoplasmic domain that could bind Lck (Collins et al., 1992). Recent results suggest that this induced redistribution of coreceptor plays a key role in coreceptor-dependent responses to antigen and that it is due in large part to protein–protein interactions mediated by the SH2 domain of CD4-associated Lck (Xu and Littman, 1993). Thus, CD4-associated Lck can function even in the absence of kinase activity, but this activity requires an intact SH2 domain. Moreover, deletion of the entire catalytic domain of CD4-associated Lck results in even higher coreceptor activity. These results suggest that the SH2 domain of CD4-associated Lck has a critical function that is masked in the presence of the catalytic domain, possibly owing to intramolecular interactions mediated by the C-terminal regulatory tyrosine (see discussion on CD45 and Csk function below). When accessible, the SH2 domain of Lck may interact with phosphorylated components of the TCR or with TCR-associated cytoskeletal elements. This might aid in anchoring CD4 and the stimulated TCR within the same oligomeric complex.

Thus, Lck can provide two functions: the kinase activity (which may or may not be associated with CD4) participates in the initial events of TCR activation by phosphorylating ARAMs; as the stimulated-state of the TCR complex matures, the SH2 domain of CD4-associated Lck is engaged by a tyrosine-phosphorylated residue(s) on a molecule(s) within the complex and this then serves to anchor CD4/Lck to the stimulated TCR complex. In this way, the efficiency of antigen recognition is potentiated by localizing more Lck kinase to the TCR complex and by increasing the avidity of the TCR–MHC molecule interaction. This is consistent with the observation that CD8 avidity for class I MHC molecules can be increased by TCR stimulation

(O'Rourke et al., 1990), although the requirement for Lck in this phenomenon has not been examined.

It is not clear whether coengagement of the TCR with CD4 or CD8 results in distinct developmental signals. There does not appear to be a requirement for TCR-mediated signal discrimination in commitment of thymocytes to the CD4 or CD8 lineage, since there is good evidence that this occurs stochastically and is followed by coreceptor-assisted TCR signaling (Chan et al., 1993; Davis et al., 1993; van Meerwijk and Germain, 1993). In this context, coreceptor function need not involve associated Lck, since a form of CD4 lacking its cytoplasmic domain and unable to bind Lck could effectively rescue T helper cell development in CD4-deficient mice. This required overexpression of the tailless CD4 molecule, consistent with the notion that CD4 functions to stabilize the TCR–MHC interaction (Killeen and Littman, 1993). Coreceptors do, however, contribute to signals that discriminate between positive and negative selection of thymocytes. Overexpression of CD8 transgenes in thymocytes bearing a specific T cell receptor that interacts with host MHC class I molecules and normally directs positive selection of the cell results in intrathymic death of the cell (Lee et al., 1992; Robey et al., 1992). Thus, the signaling threshold for negative selection is exceeded when additional CD8 molecules increase TCR–MHC avidity, the concentration of Lck, or both, within the TCR complex. Consistent with this finding, absence of CD8-MHC class I interactions permits survival of a considerable fraction of T lymphocytes with receptors for self antigen that would otherwise be deleted in thymic development (Wallace et al., 1993). How increased avidity modulates the quality of the transduced signal is not yet understood.

Coreceptor Function in B Cells

A coreceptor function in B lymphocytes is less clearly understood, but it is becoming increasingly clear that there are mechanisms for amplification of signals received by the BCR. This likely involves a molecular complex formed by CD19 and CD21 (complement receptor 2, [CR2]), as well as additional proteins such as TAPA-1 (for reviews see Fearon, 1993; van Noesel et al., 1993) (Figure 3). No ligands have yet been identified for CD19 or TAPA-1. However, CD21, binds the complement proteolytic product C3dg. The CD19/CD21 complex cocaps with the BCR complex after anti-IgM treatment. Moreover, antibody cross-linking of CD19 to the BCR lowers the threshold for receptor stimulation (Carter and Fearon, 1992). However, independent ligation of CD19 inhibits surface IgM-mediated B cell activation. These findings are remarkably similar to the properties of the T cell coreceptors, CD4 and CD8, as discussed above.

In T cells, the coreceptors and the TCR are brought into proximity through their interaction with the same MHC molecule. A similar physiological mechanism for cross-linking of the surface BCR with CD19/CD21 may be achieved by binding to immune complexes containing both antigen and complement proteolytic fragments that bind to CD21. In T lymphocytes, the coreceptor stabilizes the TCR–antigen interaction and supplies Lck to the signal transducing machinery of the receptor. It has been proposed that, likewise, the CD19/CD21 complex delivers another Src family PTK, Lyn, to the BCR complex upon cross-linking (van Noesel et al., 1993). In addition, anti-Ig-mediated B cell signaling is accompanied by tyrosine phosphorylation of CD19 and by its subsequent association with PI 3′ kinase via the consensus motif YXXM (Tuveson et al., 1993), which can associate with the SH2 domains of PI 3′ kinase. Activation of PI 3′ kinase has been previously demonstrated upon BCR ligation (Gold et al., 1992), but has been difficult to detect following TCR stimulation.

Regulation of Antigen Receptor Signal Transduction by the Protein Tyrosine Phosphatase CD45

Protein-tyrosine phosphorylation can be induced by stimulating the function of PTKs, but it can also be induced by inhibiting PTPases. The various isoforms of the CD45 PTPase, 180–220 kd proteins derived by alternative splicing, are abundant plasma membrane proteins that are differentially expressed on all cells of the hematopoietic lineage except mature erythroid cells (Trowbridge, 1991). B cells express a 220 kd isoform containing all of the products of the differentially spliced exons. On T cells, various isoforms distinguish T helper cell subsets (Bottomly et al., 1989) and resting or activated T cells (Byrne et al., 1988). Physiological ligands of CD45 have not been definitively established, but CD45 interacts with the B cell antigen CD22 (Stamenkovic et al., 1991), which binds to many plasma membrane proteins that are sialylated (Sgroi et al., 1993).

Genetic studies of T or B cell lines deficient in CD45 expression demonstrate that CD45 is required for TCR and BCR signal transduction, including the induction of PTK activity (Justement et al., 1991; Koretzky et al., 1990, 1991; Pingel and Thomas, 1989). The defect specifically affects early events in TCR-mediated signaling, since the IL-2 receptor and a heterologous receptor that, unlike the TCR, activates phospholipase C activity via a guanine nucleotide-binding (G) protein, are still functional in CD45-deficient cells (Koretzky et al., 1990; Pingel and Thomas, 1989). In mice in which the CD45 gene has been disrupted, T cell development is impaired at the CD4/CD8 double positive stage and a reduced number of T cells are detected in the periphery, whereas B cells lacking CD45 develop normally (Kishihara et al., 1993). However, both the B and T cell antigen receptor–mediated responses are impaired in these animals, despite some leakiness in CD45 expression in the T cell compartment, demonstrating that CD45 has a critical role in both BCR- and TCR-mediated signal transduction in vivo.

Studies in T cells suggest that a target of the CD45 PTPase is the negative regulatory site of tyrosine phosphorylation in Lck, in Fyn, or in both. All PTKs of the Src family contain a C-terminal tyrosine that, when phosphorylated, is associated with diminished kinase function (Cooper et al., 1986). Negative regulation of kinase function may be a consequence of an intramolecular interaction of this phosphorylated residue with the SH2 domain of the PTK. In CD45-deficient cells, Lck and, to a lesser extent,

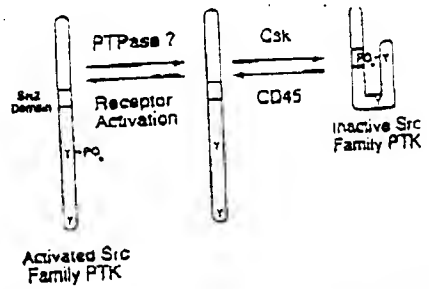

Figure 4. The Regulation of Src PTKs by the CD45 PTPase and Csk PTK

Table 1. Antigen Receptor Induced Tyrosine Phosphoproteins

	T Cells	B Cells
Plasma Membrane Proteins	TCR: CD3 δ,ε,γ	BCR Igα
	ζ	Igβ
	CD5	CD19
	CD6	CD22
Src Kinases	Lck, Fyn	Lyn, Blk, Fyn
Syk/ZAP-70 Kinases	ZAP-70, Syk	Syk
Downstream enzymes	MAP-kinases	MAP-kinases
	Vav	Vav
	PLCγ1	PLCγ1 & PLCγ2
	GAP (+/−)	GAP
		PI 3' kinase
Others	Ezrin	
	Vimentin-containing protein	

Fyn are hyperphosphorylated at this negative regulatory site (Hurley et al., 1993; Ostergaard et al., 1989; Sieh et al., 1993). Moreover, the SH2 domain of Lck in CD45-deficient cells, unlike wild-type cells, is inaccessible for binding to synthetic phosphopeptides corresponding to sequences of the negative regulatory sites (Sieh et al., 1993). CD45 can dephosphorylate this site in vitro, resulting in activation of PTK catalytic function (Mustelin et al., 1989). Thus, CD45 may dephosphorylate the negative regulatory site of critical Src members, allowing the activation of these kinases and their participation in TCR and BCR signal transduction (Figure 4). As previously discussed, this may influence the coreceptor-associated function of Lck by rendering the SH2 domain accessible. Whether CD45 or other transmembrane PTPases expressed in T and B cells have specificity for individual members of the Src family remains to be determined.

The negative regulatory sites of Src PTKs are phosphorylated by Csk, a widely expressed PTK that is itself homologous to Src PTKs (Okada et al., 1991). Overexpression of Csk in a T cell hybridoma inhibits TCR-induced protein-tyrosine phosphorylation and IL-2 production (Chow et al., 1993). Moreover, the effect of Csk overexpression can be suppressed by expressing a mutant Fyn whose negative regulatory tyrosine is changed to phenylalanine, consistent with one of the proposed Csk sites of action. Thus, the status of the phosphorylation of the negative regulatory site of the Src PTKs may be determined by the balance of activities of Csk and CD45 (Figure 4). The mechanism by which Csk function is regulated is not known.

The function(s) of the different extracellular domains of CD45 isoforms has not yet been established, but it has been suggested that ligands may regulate CD45 PTPase activity. Both stimulatory and inhibitory effects of antibodies to the various CD45 isoforms have been observed (Trowbridge, 1991). Recent studies with chimeric CD45 molecules indicate that the extracellular and transmembrane domains are not required for CD45 function (Desai et al., 1993; Hovis et al., 1993; Volarevic et al., 1993). However, studies with a chimeric receptor PTPase suggest that ligands may negatively regulate PTPase function by dimerization (Desai et al., 1993). This negative regulation may result from sequestration or dephosphorylation of the PTPase domains, since CD45 may be tyrosine phosphorylated following TCR stimulation (Stover et al., 1991).

Consequences of Antigen Receptor Induced Tyrosine Phosphorylation

Stimulation of the TCR and BCR induces the tyrosine phosphorylation of many cytoplasmic and membrane proteins. It is not yet known which of these proteins represent direct substrates of the PTKs associated with the stimu-

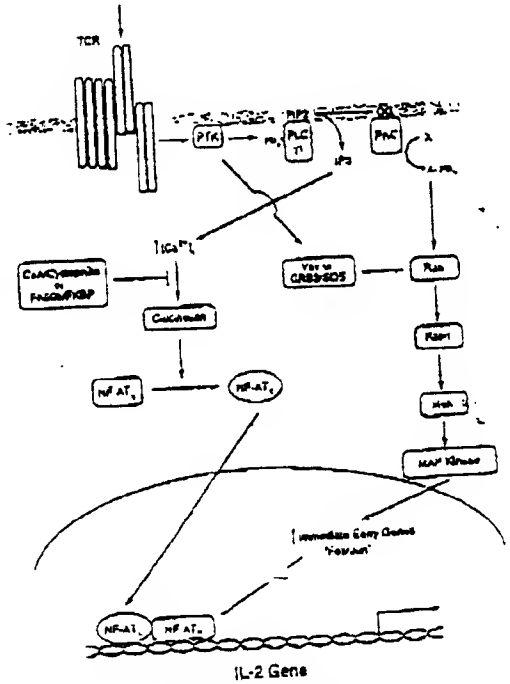

Figure 5. Downstream Signaling Pathways Induced Following TCR Stimulation

Note that the inhibition of GAP function is not depicted. The events downstream of Ras are summarized from other systems.

lated antigen receptor complex. The identity of many of these proteins and the functional significance of most of the tyrosine phosphorylations are not known. Table 1 represents a partial list of the proteins identified that are tyrosine phosphorylated in response to antigen receptor stimulation in T and B cells. We will focus on the events downstream of the TCR that have been implicated in cellular responses. The best characterized are activation of phospholipase C (PLC), calcineurin, and Ras (Figure 5). These events have been implicated in events leading to the transcriptional activation of the IL-2 gene.

Activation of the Phosphatidylinositol (PI) Second Messenger Pathway

Stimulation of the TCR induces PLC activity (Imboden and Stobo, 1985). The activation of PLCγ1 results in the hydrolysis of PI 4,5-bisphosphate yielding the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DG). These second messengers are responsible for the observed TCR-induced rapid and sustained increase in cytoplasmic free calcium ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC), respectively. The increased $[Ca^{2+}]_i$ and activation of PKC have been causally linked to cellular responses in T and B cells. The best characterized of these responses is the transcriptional activation of the IL-2 gene. The evidence supporting a role of the PI pathway in the induction of IL-2 transcription has been reviewed (Weiss and Imboden, 1987) and includes the stimulatory effects of reagents that increase $[Ca^{2+}]_i$ and activate PKC; the inhibitory effects of calcium chelators and PKC inhibitors; the ability to induce IL-2 transcription via a heterologous G protein–coupled receptor that activates the PI pathway; and the ability of activated forms of PKC or calcineurin to bypass cellular signals in inducing IL-2 gene transcription. Although the activation of the IL-2 gene in the absence of the apparent activation of the PI second messenger pathway has been reported (Sussman et al., 1988), no alternative second messenger pathways have been identified.

Biochemical and genetic data suggest that the TCR-mediated induction of PLC activity occurs by the tyrosine phosphorylation of PLCγ1 (Secrist et al., 1991; Weiss et al., 1991). PTK inhibitors prevent both the TCR-induced tyrosine phosphorylation of PLC isoforms and induction of PLC activity (Graber et al., 1992; June et al., 1990). The TCR-induced tyrosine phosphorylation of PLCγ1 is associated with its increased catalytic activity in vitro (Dasgupta et al., 1992). In mutant T cell lines selected for their failure to manifest TCR-induced PLC activation, PLCγ1 is not tyrosine phosphorylated (Weiss et al., 1991). PLCγ1 is the only tyrosine phosphorylated PLC isozyme induced following TCR stimulation, whereas both PLCγ1 and PLCγ2 isozymes are tyrosine phosphorylated in response to BCR stimulation (Carter et al., 1991; Hempel et al., 1992).

The mechanism by which the TCR phosphorylates PLCγ1 is not clear. PTK growth factor receptors interact with PLCγ1 by recruiting the enzyme via its SH2 domain to the tyrosine phosphorylated receptor. With one exception (Dasgupta et al., 1992), recruitment of PLCγ isozymes to the stimulated TCR or BCR has been difficult to detect. PLCγ1 was reported to coimmunoprecipitate with Lck and an interaction of the PLCγ1 SH2 domain with Lck was demonstrated (Weber et al., 1992). However, activation of Lck by CD4 cross-linking fails to induce the association of PLCγ1 with Lck or to induce PLCγ1 phosphorylation (Gilliland et al., 1992; Ledbetter et al., 1988). Although the phosphorylation and activation of PLCγ1 requires Lck kinase function (Straus and Weiss, 1992; Weiss et al., 1991), the interaction may be indirect.

The Calcium/Calcineurin Connection: A Link Between Plasma Membrane Signal Transduction and Nuclear Transcriptional Events

The rapid and sustained increase in $[Ca^{2+}]_i$ that results from PLC γ1 activation is thought to influence calcium/calmodulin-dependent events. An understanding of the downstream effects of the $[Ca^{2+}]_i$ increase has emerged from a remarkable series of studies aimed at elucidating the mechanism of action of the immunosuppressive drugs, cyclosporin A (CsA) and FK506. These drugs, whose clinical efficacy has revolutionized the field of organ transplantation, block immune system function, in part, by inhibiting the transcription of several lymphokine genes in T cells. Both CsA and FK506 bind to cytoplasmic proteins, known as immunophilins, forming complexes that have novel biological functions (Schreiber and Crabtree, 1992). Curiously, both cyclophilin and FKBP, the immunophilins bound by CsA and FK506, respectively, are cis-trans peptidyl-prolyl isomerases. However, the functions of the drugs are independent of their ability to block enzymatic function. Instead, the drug–immunophilin complexes interact at high affinity with calcineurin, a calcium/calmodulin-dependent serine phosphatase (PP2B) (Liu et al., 1991). The immunosuppressive activity of the drugs correlates well with their ability to inhibit calcineurin phosphatase activity. Calcineurin is expressed at low levels in T lymphocytes, hence accounting for the relative specificity of the immunosuppressive drugs in targeting T cell function. Thus, at least one critical downstream event regulated by the increase in $[Ca^{2+}]_i$ is the activation of calcineurin.

A function for calcineurin has been most firmly established in the regulation of IL-2 gene expression. Nuclear protein complexes, whose induction is sensitive to CsA and FK506, bind to two key sites within the IL-2 enhancer region upon T cell activation. These sites, antigen receptor response element 1 (ARRE-1, also known as NF-IL2A) and ARRE-2 (or the NF-AT binding site), can function independently, directing transcription from heterologous promoters upon T cell activation. Induction of both factors involves a calcium-dependent step that is sensitive to the immunosuppressive drugs. Overexpression of wild-type calcineurin renders these transcriptional responses more resistant to the drugs; in addition, their calcium dependence can be overcome by the expression of a deregulated subunit of calcineurin (Clipstone and Crabtree, 1992; O'Keefe et al., 1992). These results suggest that the phosphatase activity of calcineurin directly or indirectly contributes to the activation of these transcription factors.

NF-AT consists of CsA-sensitive and CsA-insensitive components. The CsA-sensitive component of NF-AT resides as a preformed cytoplasmic protein that is trans-

located to the nucleus upon activation. Recent studies indicate that the cytoplasmic form is a phosphoprotein of 110–140 kd, while the nuclear form is considerably smaller, possibly as a consequence of the phosphatase activity of cytoplasmic calcineurin (Jain et al., 1993). The second component of NF-AT requires induction of PKC, is newly synthesized, and appears to be a heterodimer of members of the Fos and Jun families of transcription factors (see below).

Ras Activation in T Cells

The CsA-insensitive component of NF-AT appears to be activated by a pathway involving Ras, a 21 kd GTP-binding protein with GTPase activity. Stimulation of the TCR and BCR induces a marked and rapid activation of Ras as manifested by its GTP-bound state (Downward et al., 1992). TCR-induced Ras activation is the result of both PKC-dependent and PKC-independent mechanisms that may be similar to those used by receptor tyrosine kinases. Receptor tyrosine kinases regulate Ras activity via interactions with guanine nucleotide exchange proteins, such as Sos and Vav, and GTPase activating proteins (GAPs) (Reviewed by Polakis and McCormick, 1993; Schlessinger, 1993). Both inhibition of GAP and stimulation of guanine nucleotide exchange functions have been observed following lymphocyte stimulation. GAP is tyrosine phosphorylated following BCR stimulation, but to a lesser extent following TCR stimulation (Downward et al., 1992; Gold et al., 1993). Vav, is also tyrosine phosphorylated in response to TCR or BCR stimulation (Bustelo and Barbacid, 1992; Bustelo et al., 1992; Margolis et al., 1992); the tyrosine-phosphorylated form of Vav has increased guanine nucleotide exchange activity for Ras in vitro (Gulbins et al., 1993). The guanine nucleotide exchange protein, Sos, and its adapter molecule, GRB2 (Schlessinger, 1993), are expressed ubiquitously and appear to interact with cytoplasmic PTKs. However, it is unclear whether they contribute to Ras regulation in lymphocytes.

Recently, the downstream effectors of Ras function were identified in nonlymphoid cells. Ras interacts directly with the serine/threonine kinase Raf-1, which regulates the activity of a kinase cascade that includes Mek and MAP kinase (Figure 5) (Crews and Erikson, 1993). Raf-1 is activated in T cells following TCR stimulation (Siegel et al., 1990). In T and B cells, the activation of MAP kinase has also been associated with PKC activation (Nel et al., 1990). This complex kinase cascade has been implicated in the regulation of nuclear events involved in cell growth and differentiation.

The activation of Ras has been correlated with the transcriptional activation of the IL-2 gene. Expression of an activated form of Ras, which has reduced capacity to hydrolyze GTP, can substitute, in part, for phorbol esters in synergizing with calcium ionophores to induce transcription driven by the IL-2 upstream regulatory region or a multimer of the NF-AT site (Rayter et al., 1992; Woodrow et al., 1993). Moreover, a dominant negative mutant of Ras could inhibit TCR-induced IL-2 transcription. Activated Ras is likely to be involved in the induction of the anisomycin-sensitive and calcium-independent nuclear component of NF-AT, which appears to be a heterodimer composed of Fos- and Jun-related proteins (Boise et al., 1993; Jain et al., 1992; Northrup et al., 1993), similar to that mediating AP-1 function. Thus, the integration of distinct branches of the TCR-induced signaling pathway results in the activation of distinct transcriptional regulators to induce IL-2 gene expression.

Summary

Despite the differences in the antigens that they recognize and in the effector functions they carry out, B and T lymphocytes utilize remarkably similar signal transduction components to initiate responses. They both use oligomeric receptors that contain distinct recognition and signal transduction subunits. Antigen receptors on both cells interact with at least two distinct families of PTKs via common sequence motifs, ARAMs, in the cytoplasmic tails of their invariant chains, which have likely evolved from a common evolutionary precursor. Coreceptors appear to serve to increase the sensitivity of both of these receptor systems through events that influence ligand binding and signal transduction. The critical role of tyrosine phosphorylation of downstream signaling components, such as phospholipase C, is the net result of changes in the balance of the action of antigen receptor-regulated PTKs and PTPases. The identification of downstream effectors, including calcineurin and Ras, that regulate cellular responses, such as lymphokine gene expression, promises the future possibility of connecting the complex pathway from the plasma membrane to the nucleus in lymphocytes. Insight gained from studies of the signaling pathways downstream of TCR and BCR stimulation is likely to contribute significantly to future understanding of mechanisms responsible for lymphocyte differentiation and for the discrimination of self from nonself in developing and mature cells.

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